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(54) Title: METHOD FOR PURIFYING HYDROPHOBIC PROTEINS AND PEPTIDES BY MEANS OF HYDROPHOBIC ZEOLITES

(57) Abstract

The invention relates to the purification of hydrophobic oligo-/polypeptides and/or hydrophobic proteins by means of hydrophobic zeolites, especially for analytical and preparative purposes. The purification of specific oligo-/polypeptides and/or proteins is performed by adsorption from a solution to hydrophobic zeolites in order thereafter to elute specific peptides and proteins from the hydrophobic zeolites, or to enrich in the solution the proportion of specific peptides and proteins that does not bind to the zeolites.

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METHOD FOR PURIFYING HYDROPHOBIC PROTEINS AND PEPTIDES BY MEANS OF HYDROPHOBIC ZEOLITES

The present invention relates to a method for purifying, by means of hydrophobic zeolites, hydrophobic proteins and/or hydrophobic oligo-/polypeptides, especially for preparative and analytical purposes.

Background of the Invention

Zeolites, aluminium silicates having a framework structure, have in recent years been extensively used as adsorbents of organic compounds from industrial exhaust air. This application relies on the use of hydrophobic zeolites. The degree of hydrophobicity is given by the ratio of Si/Al, where hydrophobic zeolites have a high silicon content and, hence, few structural charge carriers.

The choice of strictly hydrophobic zeolites is limited to silicalite, mordenite and zeolite Y. Generally, the usefulness of the zeolites is restricted by the size and the accessibility of the pores in the zeolite crystals. Silicalite and zeolite Y have three-dimensional pore systems and high accessibility, while the pore system of mordenite is one-dimensional and, hence, less accessible. As to the size of the pore system, both zeolite Y and mordenite are among the largest known zeolites with pore diameters of about 7 and 7.5 Å, respectively. Silicalite, on the other hand, has a pore diameter of about 5.5 Å (1).

The zeolite Y crystals cannot be made larger than 0.1-1 µm (2). This size distribution is not suitable in low-pressure chromatography, since a very high back pressure will be generated in the column. To obtain larger zeolite particles that can be used in low-pressure chromatography, the zeolite crystals can be sintered or entrapped in a carbohydrate matrix (agarose).

Purification and analysis of proteins or peptides in biochemistry and biotechnology are largely based on different chromatographic methods. Generally, the chromato-

graphic methods can be divided into gel filtration, hydrophobic chromatography, ion-exchange chromatography and affinity chromatography. Hydrophobic chromatography utilises the interaction between a hydrophobic matrix and hydrophobic proteins or hydrophobic peptides. This interaction may be due to:

- hydrophobic regions or stretches in the proteins/peptides (e.g. membrane proteins, lipases, and serum albumin)
- lipids or other hydrophobic substances covalently bonded to the proteins/peptides (e.g. the Thy-1 antigen and the decay-accelerating factor DAF CD 55)
- a hydrophobic side chain of an amino acid in the primary sequence of the proteins/peptides (e.g. the amino acids methionine, isoleucine and phenylalanine).

The matrices used today in hydrophobic chromatography are based on a strong hydrophobic interaction, and the proteins/peptides are often eluted under denaturating conditions. Hydrophobic zeolites mediate a weaker hydrophobic interaction between the matrix and the proteins/peptides. This invention is based on the use of hydrophobic zeolites as a matrix in hydrophobic chromatography. Hydrophobic zeolites have earlier been used to efficiently remove detergents from solutions containing a mixture of proteins and detergents (3,4,5). The aim has been to facilitate further purification of the proteins which would otherwise have been obstructed by the presence of the detergent.

One object of the present invention is thus to provide a method for purifying hydrophobic proteins and/or hydrophobic oligo-/polypeptides from a solution by means of hydrophobic zeolites.

Description of the Invention

The hydrophobic zeolite used according to the present invention has the composition $Na_x[(AlO_2)_x(SiO_2)_y]$, where x and y are integers and $y/x > 15$.

In an especially preferred embodiment, use is made of a zeolite where $y/x > 1000$.

Examples of usable zeolites are silicalite, mordenite and zeolite Y. The hydrophobic zeolite can be used as such or in the form of sintered zeolite crystals, or in the form of crystals entrapped or suspended in, coated or suitably combined with one or more permeable, non-zeolitic materials. One example of usable non-zeolitic materials is agarose.

Hydrophobic proteins and/or hydrophobic oligo-/poly-peptides are purified according to the invention, either by the desired protein binding completely or partly to the zeolite and being thereafter recovered, or by undesired proteins binding to the zeolite and recovering the desired protein directly. The hydrophobicity of the zeolite and the amount of zeolite to be added can be established by a person skilled in the art, and depends, inter alia, on the protein or the peptide of current interest, the composition of the solution and the desired result. In this context, "hydrophobic proteins and/or hydrophobic oligo-/polypeptides" thus means such proteins and peptides as comprise

- hydrophobic regions or stretches in the proteins/peptides (e.g. membrane proteins, lipases, and serum albumin)
- lipids or other hydrophobic substances covalently bonded to the proteins/peptides (e.g. the Thy-1 antigen and the decay-accelerating factor DAF CD 55)
- a hydrophobic side chain of an amino acid in the primary sequence of the proteins/peptides (e.g. the amino acids methionine, isoleucine and phenylalanine).

The hydrophobic proteins and/or hydrophobic oligo-/polypeptides to be purified according to the invention are present in a solution, and the method can be carried out batchwise, fully- or semi-continuously. The manner in which the zeolite and the solution containing proteins or peptides contact each other is not critical. For example,

the zeolite may be added to the solution directly, or the zeolite can be packed in a column, to which the solution is added. Where the desired protein or the desired polypeptide is adsorbed to the zeolite packed in a column, the protein or polypeptide can be e.g. eluted by displacement or by changing the three-dimensional structure of the adsorbed substance.

The amount of protein that can be adsorbed to hydrophobic zeolites increases with an increase of the Si/Al ratio of the zeolite. Example 1 below shows that the amount of immunoglobulin G (IgG) that binds to the zeolite increases by a factor 10 when the Si/Al ratio of the zeolite increases from 15 to > 1000. Different proteins have a different capacity of binding to hydrophobic zeolites, and Example 2 shows a protein that does not bind to the zeolite (horseradish peroxidase), a protein partly binding to the zeolite (albumin), and a protein binding strongly to the zeolite (IgG).

Proteins which bind to the hydrophobic zeolite can be eluted with polyethylene glycol (PEG), and the hydrophobic zeolite can thus be used, for preparative purposes, to isolate peptides and proteins.

Monoclonal antibodies (m-ab) are used in biochemistry and medical chemistry, and are isolated primarily from tissue culture supernatants. The isolation of m-ab from tissue culture supernatants is performed on a laboratory scale or on an industrial scale where hundreds of litres of tissue culture supernatants are used. Hydrophobic zeolite Y binds more than 10 mg antibodies per g zeolite, and the content of m-ab in tissue culture supernatants generally is about 10 µg/ml. Example 3 below shows that m-ab can be isolated from tissue culture supernatants by means of hydrophobic zeolite Y.

Prefractionation by precipitation with $(NH_4)_2SO_4$, ethanol or polyethylene glycol is a commonly used method for eliminating unessential proteins in connection with the purification of specific proteins. Horseradish peroxi-

dase is one of the most commonly used marker enzymes in immunochemistry. Horseradish peroxidase is yearly purified from hundreds of thousands of litres of crude extract by some form of prefractionation, followed by ion-exchange chromatography. In Example 4 below, a comparison is made between prior-art methods of purifying horseradish peroxidase and the method of the present invention. This Example shows that hydrophobic zeolite Y is an excellent alternative prefractionation method for purifying horseradish peroxidase by ion-exchange chromatography. As compared, for example, with the method using ammonium sulphate fractionation, the method of the invention is most time-saving by dispensing with additional purifying steps.

Reverse-phase HPLC is a commonly used method for analysing mixtures of peptides and proteins. The carrier material is silica which has been made hydrophobic by covalent coupling of C_2-C_{18} carbon chains. Hydrophobic zeolites with varying Si/Al ratios as carrier material in HPLC columns should provide similar analytical possibilities. However, a narrower particle size distribution is required, which is under development, before the hydrophobic zeolites can be used in HPLC columns.

Hydrophobic zeolites with varying Si/Al ratios have many potential preparative and analytical applications in biochemistry. The advantages of hydrophobic zeolites are numerous as compared with conventional hydrophobic carriers. Zeolites consist of inert material, are stable in aqueous systems within a broad pH range, completely insoluble and insensitive to oxidising and reducing agents, withstand high pressures and temperatures without changing, and are much less expensive than conventional hydrophobic carriers. The binding capacity of protein per gram of zeolite is high, and hydrophobic zeolites can be used to advantage in large-scale industrial purification processes involving peptides and proteins.

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Examples

Example 1. Adsorption of IgG to hydrophobic zeolite Y having different Si/Al ratios

Two columns were packed with one gram of sintered zeolite Y with a ratio of $y/x = 15$ and $y/x > 1000$, respectively. On the columns was applied human immunoglobulin G (IgG), 1 mg/ml, in phosphate-buffered saline (PBS). The columns were washed with PBS and eluted with 2% (w/w) polyethylene glycol 600 (PEG 600) in PBS. Flow 1 ml/min.

Si/Al ratio	Total of bound IgG (mg)	Amount of eluted IgG (mg)
15	1.4	1.2
> 1000	13.5	13.4

Conclusions:

An increased hydrophobicity of the zeolite Y is obtained with a higher ratio of Si to Al. This Example shows that an increased binding capacity of human IgG is obtained when the hydrophobicity of the zeolite is increased.

Example 2. Separation of albumin, IgG and horseradish peroxidase with hydrophobic zeolite Y

1 ml of a mixture of 1 mg human albumin, 1 mg horseradish peroxidase (HRP) and 1 mg human IgG in PBS was applied on a column consisting of 5 g sintered hydrophobic zeolite Y, y/x > 1000. The column was washed with PBS and eluted with a linear gradient of 0-20% w/w of PEG 600 in PBS. 1-ml fractions were collected. The result of the elution appears from the accompanying Table. The gradient starts in fraction 10 and ends in fraction 30.

Fraction	Peroxidase (μg/ml)	Albumin (μg/ml)	IgG (μg/ml)	Fraction	Peroxidase (μg/ml)	Albumin (μg/ml)	IgG (μg/ml)
1	-	-	-	18	-	-	-
2	-	-	-	19	-	-	-
3	-	-	-	20	-	-	-
4	-	-	-	21	-	-	-
5	-	-	-	22	-	-	-
6	88	88	-	23	-	-	-
7	300	188	-	24	-	-	-
8	277	166	-	25	-	-	-
9	174	117	-	26	-	-	68
10	108	97	-	27	21	57	308
11	50	53	-	28	18	52	278
12	-	-	-	29	-	36	190
13	-	-	-	30	-	26	139
14	-	-	-	31	-	21	109
15	-	-	-	32	-	-	88.
16	-	-	-	33	-	-	-
17	-	-	-	34	-	-	-

Conclusions:

In this Example, a mixture of proteins containing proteins of both plant and mammalian origin was prepared

in the laboratory and applied on a column containing zeolite Y. The Example shows:

- one protein, HRP, that is not adsorbed to the hydrophobic zeolite Y and can be found in the column filtrate
- one protein, IgG, that is adsorbed to the hydrophobic zeolite and can be eluted from the column using polyethylene glycol
- one protein, human serum albumin, that is adsorbed to some degree to the hydrophobic zeolite Y. The reason for this separation into one unbound and one bound fraction is not yet known, but may be due to a non-homogeneous albumin preparation in respect of the hydrophobicity of the protein.

Example 3. Purification of monoclonal antibodies from tissue culture supernatant with hydrophobic zeolite Y

20 ml of a tissue culture supernatant containing 10% foetal calf serum from the mouse hybrodima HB 79 (from the American Type Culture Collection) producing monoclonal antibodies (m-ab), IgG_{2a}, was diluted 1:4 with PBS and applied on a column consisting of 450 mg sintered zeolite Y, y/x > 1000, flow 2.5 ml/min. The column was washed with 10 ml PBS and eluted with 20% PEG 600 (w/w) in PBS. 1-ml fractions were collected, and the amount of m-ab in the fractions was determined by a competitive ELISA for mouse IgG, see Table. The tissue culture supernatant contained 2.5 µg m-ab/ml, and a total of 50 µg m-ab was applied on the column.

Fraction	Amount of monoclonal antibodies (μ/ml)
1	0
2	0
3	0
4	2.5
5	5.0
6	7.5
7	8.7
8	7.5
9	7.0
10	5.0
Elution, total	43.2

Conclusions:

This Example shows how a protein, IgG, in a complex protein solution can be adsorbed to a hydrophobic zeolite Y and eluted from the zeolite.

Example 4. Prefractionation of horseradish peroxidase (HRP) from a horseradish homogenate with hydrophobic zeolite Y and subsequent purification by ion-exhange chromatography

Horseradish was homogenised in deionised water. After centrifuging 20,000 x g for 15 min, peroxidase was purified from the supernatant by ion-exchange chromatography on CM-Sepharose. For a comparison with this purification method, two different prefractionation methods were adopted before the ion-exchange chromatography step:

A. Ammonium sulphate fractionation having the horseradish homogenate where the fraction of a saturation of 35-90% $(NH_4)_2SO_4$ is kept and applied on CM-Sepharose.

B. Adsorption to hydrophobic zeolite Y where the horseradish homogenate is applied on a column of sintered hydrophobic zeolite Y, y/x > 1000 and the column filtrate is kept and applied on CM-Sepharose.

The purity of the peroxidase is stated as specific activity (units/mg) and as RZ value, which is the quotient between absorbance 403 nm and absorbance 275 nm.

Purification method	Amount HRP (µg)	RZ value	Specific activity (Units/mg)	Yield (%)
Crude extract	578	0.2	126	100
$(NH_4)_2SO_4$ fractionation	424	0.2	125	73
Zeolite Y filtrate	281	0.4	258	49
Crude extract and ion-exchange chromatography	195	0.8	354	34
$(NH_4)_2SO_4$ fractionation and ion-exchange chromatography	204	0.8	265	35
Zeolite Y filtrate and ion-exchange chromatography	155	1.3	448	27

Conclusions:

This Example shows how non-relevant proteins in a crude protein extract can be removed from the solution by adsorption to hydrophobic zeolite Y. Removal of non-relevant proteins from crude solutions simplifies further purification of the desired protein, in this case purification of horseradish peroxidase (HRP) using ion-exchange chromatorgraphy.

Zeolite Y filtrate + ion-exchange chromatography gives a yield which is equivalent to that of ammonium sulphate + ion-exchange chromatography, but a considerably higher purity (specific activity and RZ value).

CLAIMS

1. A method for purifying hydrophobic proteins or hydrophobic oligo-/polypeptides, c h a r a c t e r i s e d by contacting a hydrophobic zeolite with a solution of one or more hydrophobic proteins or hydrophobic oligo-/polypeptides.

2. A method as claimed in claim 1, c h a r a c t e r i s e d in that the hydrophobic zeolite is in the form of sintered zeolite crystals entrapped or suspended in, coated or suitably combined with one or more permeable, non-zeolitic materials.

3. A method as claimed in claim 1, c h a r a c t e r i s e d in that it is carried out batchwise, fully- or semi-continuously.

4. A method as claimed in claim 1, c h a r a c t e r i s e d in that the hydrophobic zeolite is packed in a column, to which a solution of one or more hydrophobic proteins or hydrophobic oligo-/polypeptides is added.

5. A method as claimed in claim 1, c h a r a c t e r i s e d in that the hydrophobic zeolite is added directly to the solution.

6. A method as claimed in claim 1, c h a r a c t e r i s e d in that hydrophobic proteins or hydrophobic oligo-/polypeptides are adsorbed to a hydrophobic zeolite and thereafter eluted for preparative purposes by displacement or by changing the three-dimensional structure of the adsorbed substances.

7. A method as claimed in claim 1, c h a r a c t e r i s e d in that hydrophobic proteins or hydrophobic oligo-/polypeptides are adsorbed to a hydropbobic zeolite in order, for preparative purposes, to increase the purity of proteins or oligo-/polypeptides present in the solution which is filtered off from the zeolite.

8. A method as claimed in claim 1, c h a r a c t e r i s e d in that the hydrophobic zeolite added has the composition $Na_x[(AlO_2)_x(SiO_2)_y]$, where x and y are integers and $y/x > 15$.

9. A method as claimed in claim 1, c h a r a c t e r i s e d in that the hydrophobic zeolite added has the composition $Na_x[(AlO_2)_x(SiO_2)_y]$, and x and y are integers and $y/x > 1000$.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC5: B01D 15/00, C07K 3/20, B01J 20/18
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: B01D, C07K, B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5108617 (HÅKAN ERIKSSON ET AL), 28 April 1992 (28.04.92), column 2, line 51 - line 59; column 3, line 62 - column 4, line 2	1-9
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A	FR, A1, 2605237 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE), 22 April 1988 (22.04.88), page 4, line 14 - line 30, abstract	1-9
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A	EP, A2, 0273756 (ALUMINUM COMPANY OF AMERICA), 6 July 1988 (06.07.88), page 3, line 7 - line 10; page 4, line 6 - line 11	1-9
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Information on patent family members

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